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IFNγ-dependent interactions between ICAM-1 and LFA-1 counteract Prostaglandin E2-mediated inhibition of antitumor CTL responses.

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Running Title: Counteracting PGE_2-mediated suppression of antitumor CTL.

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Abstract

Tumor-expressed ICAM-1 interaction with LFA-1 on naïve tumor-specific $CD8^+$ T cells not only stabilizes adhesion, but in the absence of classical B7-mediated costimulation, is able to provide potent alternative costimulatory signaling resulting in the production of antitumor cytotoxic T lymphocyte (CTL) responses. This study shows that overproduction of prostaglandin (PG) E_2 by metastatic murine renal carcinoma (Renca) cells inhibited direct priming of tumor-specific CTL responses *in vivo* by preventing the IFNγ-dependent upregulation of ICAM-1 that is vital during the initial priming of naïve $CD8^+$ T cells. The addition of exogenous IFNγ during naïve $CD8^+$ T-cell priming abrogated PGE_2-mediated suppression, and overexpression of ICAM-1 by tumor cells restored IFNγ production and proliferation amongst PGE_2-treated tumor-specific $CD8^+$ T cells; preventing tumor growth *in vivo*. These findings suggest that novel anticancer immunotherapies, which increase expression of ICAM-1 on tumor cells, could help alleviate PGE_2-mediated immune-suppression of antitumor CTL responses.

Introduction

Strengthening tumor-specific $CD8^+$ cytotoxic T lymphocyte (CTL) responses is one of the most promising recent strategies in cancer therapy. However, antitumor CTL activity is widely suppressed within the tumor microenvironment. Understanding mechanisms of suppression and ways to overcome them, are key challenges in cancer immunotherapy (1-6).

Overproduction of PGE_2 by tumor cells as a result of overexpression of cyclooxygenase (COX)-2 (7-11), suppresses antitumor immune responses. Studies have shown that PGE_2 suppresses both innate and adaptive immune responses. It alters NK cell effector function by decreasing TNFα and IFNγ production (12), and reduces phagocytosis by macrophages (13). PGE_2 can also disrupt antigen presentation by dendritic cells (DC), either by inhibiting maturation, which alters the profile of cytokine production and reduces secretion IL2 and IL12, or by inducing exhaustion, which prevents the induction of CTL, type 1 helper T (Th1) cells, and natural killer (NK) cells in favor of Th2 responses (14-16).

In earlier studies from our laboratory, murine renal cell carcinoma (Renca) cells that expressed the hemagglutinin (HA) protein from influenza virus A/PR/8/H1N1 (PR8) as a tumor-specific neoantigen (RencaHA cells) were generated. We transfected them to overexpress COX-2 (RencaHA/T3 cells), which resulted in elevated PGE_2 production. Overexpression of COX-2 by RencaHA/T3 cells induced metastasis to the tumor draining lymph nodes (TDLN). Yet despite tumor growth, when $CD8^+$ T cells transgenic for the CLR TCR (a H-$2K^d$/HA–specific T cell receptor) were adoptively transferred into RencaHA/T3 tumor-bearing mice CLR T cells in the TDLN

remained naïve (15)., The lack of CL4 T cell proliferation and CTL effector function was dependent on COX-2 overexpression by RencaHA/T3. Treatment of RencaHA/T3 tumor-bearing mice with the selective COX-2 inhibitor NS-398 restored both proliferation and CTL effector function to CL4 T cells within the TDLN to the same level found within the TDLN of COX-2 negative RencaHA tumor-bearing mice.

The immunosuppressive effects of PGE_2 are known to be associated with increased intracellular cyclic adenosine monophosphate (cAMP) (17)(13), which modulates the effector function of T cells and inhibits the stabilization of the immunological synapse formed through LFA-1–ICAM-1 interactions (17-20), which influences subsequent IFNγ-dependent T-cell proliferation. Several studies have shown that ~~T-cell~~ surface expression of ICAM-1 by tumor cells can be induced by IFNγ derived from $CD8^+$ T cells during productive activation (21, 22). Therefore, we hypothesized that suppression of IFNγ production by CL4 CTL interacting with metastatic RencaHA/T3 in the presence of high concentrations of PGE_2, would inhibit upregulation of ICAM-1 on tumor cells, which is required to drive proliferation and differentiation of tumor-specific CTL responses.

In this report we demonstrate that the concentration of PGE_2 during $CD8^+$ T-cell/tumor cell interactions plays an essential role in determining the outcome of the response; shifting from productive activation of CTL at low concentrations, towards antigen-specific tolerance induction at high concentrations. We show that exogenous PGE_2 prevented the direct priming of CL4 CTL responses *in vitro* by suppressing IFNγ production by CL4 T cells when they initially interacted with RencaHA tumor cells, a critical event for proper priming. We also show that

suppression by PGE_2 was temporary and could be mitigated by increasing cell-surface expression of ICAM-1 by tumor cells. ICAM-1 expression not only drove tumor-specific $CD8^+$ T-cell proliferation, but also limited tumor growth *in vivo*.

Materials and Methods

Mice. Thy1.1$^{+/+}$ CL4 TCR–transgenic BALB/c mice (6 to 8-wk-old) (23), and Thy1.2$^{+/+}$ BALB/c mice were maintained under specific pathogen–free conditions at the University of Bristol Animal Services Unit. Some BALB/c mice were injected subcutaneously (s.c.) with 1×10^6 Renca tumor cells and tumor growth was assessed as previously described (15, 21).

Cell lines. COX-2-overexpressing RencaHA/T3 cells (9, 21, 24), were transfected with 1.5 µg of ICAM-1 expressing pIREShyg plasmid (a kind gift from Prof. Adrian Whitehouse at Leeds University), to generate RencaHA/T3/ICAM-1. Renca cells were grown in a complete medium (RPMI (1640); Sigma-Aldrich, Poole, UK) supplemented with 10% vol./vol. fetal calf serum (FCS, Invitrogen, Paisley, U.K.); 2 mM L-glutamine (Invitrogen); penicillin/streptomycin (50 U/ml, Invitrogen), 5×10^{-5} M 2-mercapto-ethanol (Invitrogen). Medium was further supplemented with; geneticin (100 µg/ ml, Invitrogen) for RencaHA cells, plus puromycin (1 µg/ml; Biomatik, Wilmington, USA) for RencaHA/T3, plus hygromycin B (250 µg/ml, Invitrogen) for RencaHA/T3/ICAM-1. Expression of HA, COX-2, ICAM-1 and concentrations of PGE_2 were routinely monitored during the study.

Enrichment of K^d/HA-specific CL4 $CD8^+$ T cells. Single cell suspensions from peripheral lymph nodes and spleen of Thy1.1$^+$ CL4 TCR–transgenic mice were enriched for $CD8^+$ T cells using anti-CD8 MACS midiMACS (Miltenyi Biotec, Bisley, UK) (25).

CFSE labeling. MACS-purified naïve CL4 $CD8^+$ T cells were pelleted, resuspended in 50×10^6 cells/ml in PBS and mixed with 5 µM CFSE (BioLegend, San Diego, USA), for 15 minutes at 37ºC in the dark, and then washed with 45ml complete medium.

Priming of naïve CL4 $CD8^+$ T cells by tumor cells. Naïve CFSE-labeled CL4 $CD8^+$ T cells were cultured with irradiated tumor cells at a ratio of 1:1 in the presence of different concentrations of PGE_2 (Sigma-Aldrich). In some experiments, RencaHA cells were treated overnight with different concentrations of PGE_2, before being washed and used for priming naïve CL4 cells. In other experiments, CL4 cultures were treated with recombinant (r)IFNγ (Peprotec), or cultured in plates coated with rICAM-1 (Sion Biological Inc.), overnight before adding RencaHA and CL4 cells.

Priming of naïve CL4 $CD8^+$ T cells with plate-bound monoclonal antibodies (mAb). Tissue culture plates were coated with 10 mg/ml anti-CD3 (eBioscience, Hatfield, U.K.) at 4°C. Control wells contained PBS only. MACS-purified, CFSE-labeled naïve CL4 cells (5×10^4 – 25×10^4) were cultured, with or without CD28 mAbs (5 µg/ml, eBioScience), for 48 at 37°C with 5% vol./vol. CO_2. CL4 cells were then collected and stained with various other mAbs. In other experiments, plates were coated with CD3 mAbs and/or rICAM-1 before the addition of naïve CL4 cells. For the secondary activation of CL4 $CD8^+$ T cells pre-activated by tumor, CFSE-labeled CL4 cells (1×10^6) recovered from primary cultures were restimulated with 1×10^5 freshly irradiated tumor cells in 24 well plates in the presence or absence of 10^{-6} M PGE_2 for 37°C in 5% vol./vol. CO_2.

cAMP ELISA. CL4 cells were treated with 1 mM of 3-Isobutyl-1-methylxanthine (IBMX), an inhibitor of cAMP phosphodiesterases, for 1 h at 37°C followed by 10^{-6} M PGE_2 for 10mins, then lysed at 4°C. ELISA was performed using the BIOMOLFormat A Cyclic AMP plus EIA kit (Exeter, UK), according to the manufacturer's instructions. Plates were read at 405 nm with a 590 nm reference using a 3550 microplate reader (BioRad, CA, USA). Data were analyzed using Microplate Manager® 4.0 (BioRad) and the graphs plotted using Prism 4.03 software (GraphPad).

Flow cytometry. MACS-purified CL4 cells were restimulated *in vitro* for 4 h in the presence of 1µg/ml of both K^dHA peptide and Golgiplug (BD Bioscience San Diego, USA), stained with Zombie Aqua™ to exclude dead cells (Biolegend), and various fluorescently-conjugated mAbs against surface markers; CD69, CD62L and CD44 (BioLegend). CL4 cells were then permeabilized using BD Perm/fix kit (BD Bioscience) according to manufacturer's instructions and stained with anti-IFNγ mAbs (Biolegend). Cells were analyzed using an LSRII or FACSCalibur flow-cytometer with DiVa or CellQuest software respectively (BD Cytometry Systems Oxford, UK).

Results

To determine if PGE_2 affected the direct priming of naive tumor-specific $CD8^+$ T cells by tumor cells, CFSE-labeled naïve K^d/HA-specific CL4 $CD8^+$ T cells were cocultured with RencaHA cells for 72 h in the presence of increasing concentrations of PGE_2 ([RencaHA+CL4]+PGE_2; Fig, 1A, top). Nontransfected (HA-negative), RencaNT cells were used as a negative control. After coculture, CL4 cells were isolated and analyzed for proliferation using standard ^{3}H-thymidine incorporation (Fig. 1, left), as

well as for intracellular IFNγ expression using flow cytometric analyses (Fig. 1, right). In the absence of PGE_2, CL4 cells proliferated and produced IFNγ production compared with CL4 cells cocultured with RencaNT cells. However, in the presence of 10^{-6}-10^{-10} M PGE_2, proliferation and IFNγ production by CL4 cells was significantly reduced when compared with untreated cultures and at 10^{-11}M PGE_2 CL4 proliferation increased in response to RencaHA cells; although this was not significant. Importantly, when RencaNT cells were cultured with CL4 cells in the presence of PGE_2, no significant change was observed amongst CL4 cells and they maintained their naïve phenotype (data not shown). Correlating with reduced proliferation, amongst CL4 cells cultured with RencaHA cells in the presence of the highest concentrations of PGE_2, (10^{-6} and 10^{-7} M), IFNγ expression was significantly reduced compared with PGE_2-untreated cultures. However, when CL4 cells were cultured with RencaHA at lower concentrations of PGE_2, (10^{-10} M), both proliferation and IFNγ expression increased compared with untreated cultures.

The regulation of naïve $CD8^+$ T-cell responses by PGE_2 could be direct, or indirect,, by conditioning the tumor cells. To determine if the effect is indirect, RencaHA cells were pretreated with various concentrations of PGE_2 (Fig. 1A, bottom). However, prior to coculture, RencaHA cells were washed to remove any excess PGE_2 ([RenaHA+PGE_2]+CL4). After 72 h CL4 cells were isolated and analyzed for proliferation (left panel) and intracellular IFNγ expression by flow cytometry (right panel). The data show that, compared with PGE_2-untreated cultures, pretreatment of RencaHA cells with PGE_2 did not result in a significant reduction in CL4 proliferation even at the highest concentration of PGE_2. Furthermore, IFNγ expression was also unaffected by pretreatment of RencaHA cells with PGE_2, with a slight increase in

expression being significant only at 10^{-8} M. Together these data suggest that high concentrations of PGE_2 suppresses proliferation and CTL effector function *in vitro* by acting directly upon CL4 cells.

The direct effect of high concentrations of PGE_2 on CL4 cell priming was further examined by culturing CFSE-labeled CL4 cells in the presence of immobilized anti-CD3 and anti-CD28 mAb with or without 10^{-6} M PGE_2 (Fig. 1B). Although untreated CL4 T cells proliferated and elaborated IFNγ, the presence of high concentrations of PGE_2 resulted in a reduction in proliferation, with many more undivided CFSE-high cells, and a significant decrease in IFNγ expression. Thus, the inhibitory effects of PGE_2 are most likely mediated through direct action on naïve CL4 cells.

PGE_2 inhibits reactivation of $CD8^+$ T cells

To determine whether or not inhibition of CL4 T-cell proliferation and IFNγ production by high concentrations of PGE_2 is permanent, naïve CFSE-labeled CL4 cells were first primed *in vitro* with RencaHA cells in the presence or absence of PGE_2. After 48 h, CL4 cells were isolated and washed to remove excess PGE_2, before undergoing a secondary culture for a further 72 h with fresh RencaHA cells in the presence or absence of more PGE_2. CL4 cells cultured in the presence of nontransfected, HA negative RencaNT cells were used as a control.

The data show that CL4 cells from primary cocultures without PGE_2 proliferated and produced IFNγ (Fig. 2A; top row), and following secondary coculture with fresh RencaHA cells alone in the absence of PGE_2, resulted in further proliferation with over than 40% of cells being $IFN\gamma^+$. However, when PGE_2 was added to these

secondary cocultures, proliferation was less, with fewer than 10% of cells being IFNγ$^+$. Critically, when PGE_2-treated CL4 cells from primary cocultures underwent a secondary coculture with fresh RencaHA cells in the absence of PGE_2, nearly half of the CL4 cells had proliferated, and one-tenth of divided cells were IFNγ$^+$, as compared with CL4 cells to which PGE_2 was also added in the secondary coculture (Fig. 2A; bottom row). Therefore, suppression by PGE_2 was reversible, and withdrawing PGE_2 from the environment could restore CL4 proliferation and CTL effector function.

The inhibition of effector function among PGE_2-treated CD8$^+$ T cells is associated with increased cAMP, which inhibits IFNγ production and the acquisition of CTL effector function (17-20, 26). To establish whether or not PGE_2 suppresses naïve CL4 cells in a cAMP-dependent manner, naïve PGE_2-treated CL4 cells were lysed, and intracellular cAMP concentrations were measured using an ELISA. Following PGE_2 treatment, cAMP expression amongst CL4 cells rose by around threefold compared to untreated CL4 cells (Fig. 2B).

IFNγ reverses inhibition of CTL function

We previously demonstrated that IFNγ treatment of RencaHA cells increased antitumor CTL responses by enhancing MHC class I (H-2K^d) expression by tumor cell (21). To determine whether or not the presence of exogenous IFNγ could directly influence CL4 T-cell responses in the presence of PGE_2, CFSE-labeled naïve CL4 cells were cocultured with RencaHA cells in the presence or absence of PGE_2 and rIFNγ and proliferation, (determined by reductions in CFSE), and IFNγ production, were assessed by flow cytometry. The proliferation and IFNγ production of CL4 T

cells were inhibited by PGE_2.(Fig. 3A). However, addition of rIFNγ to these PGE_2-treated RencaHA+CL4 cocultures counteracted the suppression of proliferation and IFNγ production. Addition of rIFNγ also resulted in increased expression of the early activation marker CD69 on proliferating CL4 cells, indicating that the presence of rIFNγ, during the initial priming of naïve CL4 cells, is essential to reverse the suppressive effects of PGE_2 (Fig. 3B). Treatment of RencaNT+CL4 cocultures with rIFNγ did not result any nonspecific increase in proliferation amongst CL4 cells (Fig. 3A and B: top rows).

As IFNγ receptors are expressed on a variety of cell types including naïve $CD8^+$ T cells and tumor cells (27, 28), it is possible that in the RencaHA+CL4 cocultures, IFNγ may be acting on either or both cell types. To address this issue, naïve CFSE-labeled CL4 cells were primed with mAb to CD3 and CD28 in the presence or absence of PGE_2 with or without rIFNγ. As anticipated control mAb-primed CL4 cells elaborated IFNγ after 72 h, which was further enhanced by the addition of rIFNγ (Fig. 3C). However, although after PGE_2 treatment, IFNγ production by CL4 cells primed with mAbs to CD3 and CD8 was inhibited, addition of rIFNγ did not restore IFNγ production by CL4 cells. Therefore, these data clearly show that reversal of the PGE_2-mediated suppression of CTL effector function by IFNγ can only occur in the presence of Renca-HA cells.

To test whether or not rIFNγ counteracts PGE_2-mediated suppression by acting directly on RencaHA cells, cocultures were set up in which RencaHA cells were treated with rIFNγ at different time points in the presence of PGE_2 (Fig. 4). Addition of 10 ng/ml of rIFNγ to RencaHA+CL4 cocultures at 0 and 20 h resulted in an

increase in proliferation, as both CFSE and IFNγ were reduced (Fig. 4; middle two rows). However, addition of rIFNγ to the cocultures after 40 h of exposure to PGE_2 did not enhance IFNγ production by CL4 cells (Fig. 4; bottom row). This suggests that the reversal of PGE_2-mediated suppression by rIFNγ can occur in the very early stages of CL4 T-cell priming.

ICAM-1–LFA-1 interactions abrogate inhibition in vitro

Not only is ICAM-1 interaction with T cell–expressed LFA-1 crucial for the homotypic T-cell aggregation that is required for T-cell communication and exchanging information (29), but the LFA-1–ICAM-1 interaction can transduce downstream costimulatory signals and drive T-cell proliferation (6, 27). We have shown that the cell-surface expression of ICAM-1 by RencaHA cells is crucial for direct priming of naïve CL4 T cells. Moreover, we showed that upregulation of ICAM-1 expression is induced by IFNγ derived from CL4 cells during their early activation by RencaHA cells. This reinforces further proliferation and the induction of CTL effector function (21, 22). Based upon these findings, we wished to compare classical anti-CD28 costimulation, with alternative ICAM-1-mediated costimulation through LFA-1 Fig. 5A). In the presence of rICAM-1, proliferation of naive CL4 T cells was significantly greater compared with the proliferation in the presence of anti-CD28 mAbs. Whereas some CL4 cells proliferated in response to CD3 mAb alone, rICAM-1 alone did not induce any proliferation (Fig. 5A). These results indicated that ICAM-1 acts as a highly potent alternative costimulatory molecule to drive naïve CL4 T-cell proliferation.

It is known that following priming, T cells decrease surface expression of CD62L, and increase CD44 expression, enabling extravasation through blood vessels into inflamed tissues and the formation of effector memory T (T_{em}) cells (28). To test whether or not classical (CD3 mAb + CD28 mAb) or alternative (CD3 mAb + rICAM-1) priming of naïve CL4 cells *in vitro* gave rise to T_{em} cells, CFSE-labeled naïve CL4 cells were primed accordingly and the expression of CD62L, CD44, and IFNγ was assessed by flow cytometry. Treatment of CFSE-labeled CL4 cells with anti-CD3 + rICAM-1 consistently produced more IFNγ-expesssing cells that divided two or more times, than when CL4 cells were primed with anti-CD3 + anti-CD28, (Fig. 5B),. Therefore, the induction of CTL effector function is at least comparable. Given the high expression of CD44 and low expression of CD62L under both conditions, the cells are T_{em} CTL (Fig. 5B).

Our previous studies showed that, although low expression of ICAM-1 by RencaHA cells is sufficient to prime naïve CL4 cells (21), PGE_2 has the ability to prevent priming of CL4 cells *in vitro* and *in vivo* (15). Therefore, we wished to compare the effect of PGE_2 on classical (CD3 mAb + CD28 mAb), with alternative (CD3 mAb + rICAM-1) priming of CL4 cells *in vitro.* Furthermore, we also wished to determine whether or not increasing ICAM-1–mediated costimulation, by the addition of rICAM-1, could counteract PGE_2-mediated inhibition of naïve CL4 T-cell priming.

Addition of exogenous PGE_2 has a greater inhibitory effect upon classical CD28-mediated costimulation compared with alternative ICAM-1-LFA-1 costimulation (Fig. 6A). When CL4 cells were cocultured with RencaHA cells, addition of PGE_2 resulted reduced CL4 proliferation by about two-thirds. However, in the presence of rICAM-1

CL4 proliferation, in response to coculture with RencaHA cells, was unaffected by PGE_2 (Fig. 6B). Thus, although PGE_2 has a greater inhibitory effect upon 'classical' costimulation compared with 'alternative' costimulation pathway, suppression is significantly abrogated by the addition of rICAM-1.

Countering PGE_2 inhibition, with overexpressed ICAM-1.

It is evident that IFNγ exerts its antitumor effects by directly (27, 30) enhancing immunogenicity through elevated ICAM-1 expression (10, 31). To determine whether or not upregulation of ICAM-1 is instrumental in abrogating PGE_2-mediated suppression, COX-2-overexpressing RencaHA/T3 cells (15) were further transfected with a cDNA plasmid expressing full-length murine ICAM-1. The resulting RencaHA/T3/ICAM-1 cell line expressed much more ICAM-1 compared with conventional RencaHA/T3 cells, which increased further after treatment with rIFNγ (Fig, 7A). Despite having equivalent amounts of HA protein and PGE_2 production (data not shown), overexpression of ICAM-1 by RencaHA/T3/ICAM-1 cells increased proliferation of naïve CL4 cells *in vitro* compared with RencaHA/T3 cells (Fig. 7B). Whereas s.c. injection of RencaHA/T3 cells into BALB/c mice resulted in the formation of solid tumors after 2 weeks, s.c. injection of RencaHA/T3/ICAM-1 cells did not result in tumor growth (Fig. 7C&D). Together, these data clearly show that overexpression of ICAM-1 can counteract PGE_2-mediated immunosuppression; restoring CTL effector function and profoundly preventing tumor growth *in vivo.*

Discussion

Overexpression of COX-2 by RencaHA/T3 cells not only results in abortive activation of CL4 $CD8^+$ T cells in the TDLN of RencaHA/T3 tumor-bearing mice, but also facilitates metastasis of RencaHA/T3 cells to the TDLN (15). However, whilst metastases of RencaHA/T3 cells to the TDLN would allow direct priming of naïve CL4 cells, overproduction of PGE_2 prevented the induction of antitumor CTL responses in these mice (32). The experiments described in this report set out to determine the mechanisms of PGE_2-mediated immunosuppression.

PGE_2 exhibits various and sometimes opposing effects on the immune responses. For example, not only does PGE_2 stimulate activation of mast cells (33, 34), it also inhibits cytokine release by macrophages (35). We have shown that the concentration of PGE_2 is a major factor in determining its overall affect upon $CD8^+$ T-cells responses. At physiological concentrations of ≤ 20 ng/ml, produced by constitutive expression of COX-1 (36), PGE_2 is able to enhance productive activation of CL4 cells. However, at high concentrations found in COX-2 overexpressing tumor micro-environments *in vivo* (37), PGE_2 prevents proliferation and IFNγ production CL4 cells in response to RencaHA cells. We show that PGE_2 mediates its effects through direct action on CL4 cells. In the absence of RencaHA cells, proliferation and IFNγ production by PGE_2-treated CL4 cells primed by anti-CD3+anti-CD28 mAb was also reduced, and pretreatment of RencaHA cells with PGE_2 did not reduce proliferation or IFNγ production by CL4 cells.

The fact that the PGE_2-mediated suppression of CL4 cell responsiveness could not be reversed by the addition of IL2 (Ahmadi and Morgan unpublished data) suggests

that PGE_2 does not induce a state of functional unresponsiveness due to anergy. Our findings correlate with other data showing that PGE_2 directly inhibits CTL function such as IFNγ production (20). However importantly, PGE_2-mediated suppression of CL4 cells is reversible, as secondary re-stimulation of PGE_2-conditioned CL4 cells, with RencaHA cells in the absence of PGE_2, restored proliferation and IFNγ production. Furthermore, irrespective of the primary culture conditions, the presence of PGE_2 in the secondary cultures renders CL4 cells refractory to proliferation and IFNγ production.

Consistent with the inhibition of CL4 CTL responses was an observed increase in intracellular cAMP following treatment with PGE_2. Increased cAMP inhibits IFNγ production by T cells as well as inhibit stabilization of the interaction between LFA-1 and ICAM-1 (17, 18) thus preventing T-cell activation due to the inability of the T cells and APCs to form a stable a synapse (18, 19). Yet, we showed that CL4 cells increased cell-surface expression of the early activation marker CD69, possibly as a result of cognate TCR signaling (38). We suggest that, although some initial cognate interactions may occur that result in the upregulation of CD69, these interactions are not sustained sufficiently enough to trigger IFNγ production to promote further interactions. Our finding that rIFNγ reversed PGE_2-mediated suppression of naïve CL4 priming by directly increasing the immunogenicity of tumor cells correlates with other studies that suggest that IFNγ enhances antigen presentation by tumor cells through increased MHC class I expression (21, 22).

Previously we showed that in the absence of classical CD28-mediated costimulation, the interaction of LFA-1 with ICAM-1 provides sufficient costimulatory signals to

prime naïve $CD8^+$ T cells and induce CTL function (8, 10). We now show that costimulation provided through LFA-1 is more potent than costimulation through CD28, which may arise from that fact that the type and quantity of the signals induced within the T cell by these two costimulatory receptors are distinct from each other (29).

In the presence of rICAM-1 cultures, CL4 cells produced more IFNγ and were $CD44^{high}$ and $CD62L^{low}$, facilitating T-cell migration from LN to the site of inflammation, a typically T_{em} CTL phenotype (30). Such differences were also observed when priming naïve CL4 cells with RencaHA tumor cells, in which costimulation occurs solely through ICAM-1-LFA-1 interaction. Blocking LFA-1 or ICAM-1 causes a reduction in CTL production of IFNγ and lysis (1, 2); therefore enhancing tumor invasion and metastasis. In addition, studies have shown in breast cancer that silencing of the ICAM-1 gene by siRNA decreased tumorigenicity *in vitro* (39).

We showed that exposure to PGE_2 during priming with both classical and alternative costimulation pathways gave rise to a reduction in CL4 proliferation and IFNγ production. However, PGE_2-mediated suppression of priming with classical costimulation was greater than with alternative rICAM-1-mediated costimulation. In the presence of PGE_2, addition of rICAM-1 together with RencaHA-expressed ICAM-1 restored CL4 proliferation. Thus, in the presence of PGE_2, LFA-1–ICAM-1 signaling is essential to fully initiate CL4 T-cell priming, presumably due to the stabilizing effect of rICAM-1 on T-cell / tumor cell interactions,,increasing TCR signaling.

Increased expression of ICAM-1 among melanoma cells gives rise to an increase in the lysis-susceptibility of melanoma cells by lymphokine-activated killer cells (LAKs) (40). Other studies also showed that transfecting gastric cancer cells with ICAM-1 causes significant increases in both adhesion to PBMC and subsequent lysis (41). Induction of ICAM-1 by artificial transcription factors results in decreased growth of ovarian cancer cells (42). Studies in mice show that siRNA-mediated silencing of hepatic ICAM-1 *in vivo* prior to injecting C26 murine colon carcinoma cells alters the liver microenvironment. In ICAM-$1^{-/-}$ mice, reduced numbers of C26 cancer cells were obtained from the liver as well as myeloid suppressor cells, and the numbers of TILs were increased compared with controls (43). In our study we found that overexpression of ICAM-1 in RencaHA/T3 cells restored their ability to induce CL4 cell proliferation *in vitro,* despite the presence of PGE_2. However, although these RencaHA/ T3/ICAM-1 cells express abundant ICAM-1 post transfection, treatment with exogenous IFNγ was could further increase ICAM-1 expression. Unlike conventional RencaHA/T3 cells, RencaHA/T3/ICAM-1 cells did not form tumors in BALB/c mice, suggesting that potent antitumor CTL had been primed which eradicated the tumors.

Based upon our findings, we propose the following model to explain the role of alternative LFA-1–ICAM-1 costimulatory interactions in restoring CTL function in the presence of PGE_2 (Supplementary Fig. S1). In the steady state, naïve CL4 T cells express LFA-1 with low affinity for ICAM-1, and although coculture of naïve CL4 cells with RencaHA cells enables CL4 TCR interactions with H-$2K^d$/HA peptide complexes, this interaction is transient and may be terminated if a synapse does not

form between the interacting cells. However, this initial contact is sufficient to stimulate Ca^{2+} influx inside CL4 cell, which provides the necessary stimuli to increase the affinity of LFA-1 for ICAM-1 (Supplementary Fig. S1A). When expression of ICAM-1 on RencaHA cells is too low to maintain a stabilized interaction between CL4 cells and RencaHA cells (Supplementary Fig. S1B), increased cAMP levels within CL4 cells will interfere with Ca^{2+} influx, resulting in a reduction in the affinity of LFA-1 for ICAM-1. Eventually, interactions between the CL4 and RencaHA cells are terminated and they disassociate without any CL4 proliferation and/or IFNγ production. However, production of IFNγ in the vicinity of CL4 cells and RencaHA cells coming into contact with one and another, increases ICAM-1 expression, thus promoting further interactions between LFA-1 and ICAM-1, the formation of the synapse, and stabilized binding between these cell types. In contrast to signals mediated by through CD28 ligation, the signals associated with LFA-1-ICAM-1 interactions appear to be essential for antitumor specific $CD8^+$ T cells to overcome the inhibitory effects of PGE_2, and generate mature CTL. When expression ICAM-1 on RencaHA cells is high (Supplementary Fig. S1C), the increased affinity of LFA-1 for ICAM-1 serves to maintain the contact between CL4 and RencaHA cell, enabling the formation of a stable synapse to be formed between the two cell types. Following synapse formation, the signals provided by both TCR–K^dHA and LFA-1–ICAM-1 interactions further increase Ca^{2+} influx, inducing a high affinity state of LFA-1 for interaction with ICAM-1. In this situation, the inhibitory signals on Ca^{2+} influx in CL4 cells, induced by PGE_2-dependent increases in cAMP levels, cannot override the stimulatory signals maintained by stable TCR–K^d/HA and LFA-1–ICAM-1 interactions, such that the net result is the expansion of antitumor CTL.

In conclusion, the data presented in this report support the thesis that overproduction of PGE_2 by tumor cells that have metastasized to the TDLN favors tumor progression in the presence of an otherwise competent immune system by preventing productive activation of tumor-specific CTL responses within the TDLN. Thus, the use of PGE_2-specific inhibitors to reduce or inhibit PGE_2 production at the tumor site could promote antitumor-specific CTL responses from both naïve and pre-activated $CD8^+$ T cells. Our data also show that IFNγ-dependent upregulation of ICAM-1 expression by tumor cells protects tumor-specific CTLs from the inhibitory effect of PGE_2, by sustaining $CD8^+$ T-cell activation, proliferation, and induction of CTL effector function. Therefore, drugs that can increase the expression of cell-surface expression of ICAM-1 by tumor cells could provide us with a powerful immune-therapeutic tool to counteract the CTL-inhibitory action of tumor-derived PGE_2,, and ultimately control tumor growth.

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Figure 1: PGE_2 can inhibit or enhance the effector function of naïve CL4 cells, primed by RencaHA tumor cells. Naïve, $Thy1.1^+$ CFSE-labeled CL4 cells (1×10^6) were cultured **(A)** with irradiated RencaHA tumor cells (1×10^5) in the presence or absence of different concentrations of PGE_2 (RencaHA+CL4)+PGE_2 (top panels), or with irradiated RencaHA tumor cells (1×10^5); pretreated with different concentrations of PGE_2 overnight (RencaHA+PGE_2)+CL4 (bottom panels), **(B)** precoated with anti-CD3 (10 µg/ml) alone, or with anti-CD3 plus or minus anti-CD28 (5µg/ml) in the presence of 10^{-6} M PGE_2. Bars **(A),** show CL4 T-cell proliferation as counts per minutes (cpm) of ^{3}H-thymidine incorporation (left) and the percentage change in $IFN\gamma^+$ CL4 cells (right). **(B;** left) show CL4 T-cell proliferation as loss of CFSE *versus* IFNγ (top row) as well the number of divided cells (bottom). Numbers in the left quadrants show the percentage of CL4 cells that produce IFNγ. Bars **(B;** right) show IFNγ expression amongst $Thy1.1^+$ CL4 cells with and without PGE_2. Data are representative four separate experiments. Statistical analyses were carried out using one way ANOVA followed by Bonferroni's test, **P* <0.05, ** *P* <0.01, *** *P* < 0.0001.

Figure 2: The presence of PGE_2 in the culture is required to inhibit priming of both naïve and effector CL4 cells. **A,** Naïve Thy1.1$^+$ CFSE-labeled CL4 cells (1 x 10^6) were cultured with irradiated RencaHA cells (1 x 10^5) in the absence or presence of 10^{-6} M PGE_2 (Primary cultures) for 48 h. Primed CL4 cells (1 x 10^6) were collected from the primary cultures and co-cultured again with 1x10^5 fresh irradiated RencaHA tumor cells in the presence or absence of 10^{-6}M PGE_2 for a further 72 h (Secondary cultures). Dot plots show CL4 T-cell proliferation as loss of CFSE, together with IFNγ expression amongst Thy1.1$^+$ CL4 cells. Numbers in the left quadrants show the percentage of CL4 cells that have proliferated and/or produce IFNγ from the total CL4 population acquired. Data are representative of three separate experiments. **B,** 2x10^6 naïve CL4 cells were also cultured in the presence or absence of 10^{-6}M PGE_2. CL4 cells were lysed and the levels of intracellular cAMP determined by ELISA. Bars show mean expression of cAMP amongst CL4 cells expressed as pg/ml. Error bars represent SD. Statistical analyses were carried out using one way ANOVA. ***$P < 0.0001$. Data are representative of two separate experiments.

Figure 3: IFNγ reverses the inhibition of CTL function caused by PGE_2. $1x10^6$ naïve, $Thy1.1^+$ CFSE-labeled CL4 cells were cultured in 24 well plates with: **(A&B)** irradiated RencaHA or Renca-NT tumor cells (1×10^5), or **(C)** coated with mAbs to CD3 mAb (10 µg/ml) and CD28 (5 µg/ml). Cultures were left untreated (alone) or treated with rIFNγ (10 ng/ml) and/or 10^{-6} M PGE_2 for 72 h. CL4 cells were isolated from the cultures and analyzed by flow cytometry. Dot plots show proliferation as loss of CFSE amongst CL4 cells gated on Thy1.1 expression *versus* either **(A&C)** IFNγ or **(B)** CD69. Numbers show the percentage of CL4 cells that are in the quadrant. Data are representative of two separate experiments.

Figure 4. IFNγ is vital during the initial phases of RencaHA-mediated priming of naïve CL4 CD8$^+$ T cells. Irradiated RencaHA and Renca-NT tumor cells (1 x 10^5) were cocultured with naïve Thy1.1$^+$ CFSE-labeled CL4 cells (1 x 10^6) in the presence or absence of 10^{-6} M PGE_2. Cultures were left untreated (no rIFNγ), or treated with 10 ng/ml of rIFNγ at the beginning (0 h), and after 2 h or 40 h of the culture. At 72 h, CL4 cells were isolated from cultures and stained for intracellular IFNγ. Dot plots are gated on Thy1.1$^+$ CL4 cells, and numbers above indicate the percentage of IFNγ$^+$ CL4 cells. Data are representative four independent experiments.

Figure 5: CL4 CD8$^+$ T-cell activation with anti-CD3 along with either rICAM-1 or anti-CD28. Naïve, Thy1.1$^+$ CFSE-labeled CL4 cells (2×10^4) were cultured with either PBS or combinations of anti-CD3 (10 µg/ml), anti-CD28 (5 µg/ml) and rICAM-1 (3 µg/ml) as shown. **A:** Bars show CL4 proliferation as counts per minute (cmp) from ^{3}H-thymidine incorporation at 48 and 72 h for each condition and error bars represent standard deviation (SD). Data are collected from three independent experiments. Statistical analyses were carried out using one way ANOVA followed by Bonferroni's test ***$P < 0.0001$. **B:** Histograms (bottom) show proliferation as loss of CFSE labeling, and dot plots (upper) show expression of CD44 and CD62L and IFNγ. Numbers in each dot plot show percentage of proliferated cells expressing each marker. Data is representative of two separate experiments.

Figure 6: The effect of rICAM-1 on CL4 CD8$^+$ T-cell proliferation in the presence of PGE$_2$. Naïve, Thy1.1$^+$ CL4 cells (2 × 10^4) were cultured in 96 well plates: **(A)** with either PBS or combinations of anti-CD3 (10 µg/ml), anti-CD28 (5 µg/ml) and rICAM-1 (3 µg/ml), in the presence or absence of 10^{-6} M PGE_2 as shown, or **(B)** with different concentrations of rICAM-1 plus 1 ×1 0^4 irradiated RencaHA cells or Renca-NT cells and 1 × 10^4 purified naïve CL4 CD8$^+$ T in the presence or absence of 10^{-6} M PGE_2. Bars show mean proliferation of CL4 cells at 48h for each condition as counts per minute (cpm) of ^{3}H-thymidine incorporation, and error bars represent standard deviation (SD). Statistical analyses were carried out using one way ANOVA followed by Bonferroni's test, *P < 0.05, ***P < 0.0001. Data is representative of four separate experiments.

Figure 7: Overexpression of ICAM-1 by PGE_2-expressing tumor cells restores CL4 proliferation* in vitro *and inhibits tumor growth* in vivo*.

A: Renca-HA/T3 and Renca-HA/T3/ICAM-1 cells were cultured in the presence or absence of 10ng/ml of IFNγ for 48 h, then stained for ICAM-1. Histograms show anti-ICAM-1 mAb staining of non-treated cultures (solid) and the IFNγ-treated cells (dashed). Filled line represents isotype control mAb staining of non-IFNγ-treated cells. **B:** Irradiated Renca-HA/T3 and Renca-HA/T3/ICAM-1 cells were also cultured with 1 x 10^4 naïve CL4 cells at a ratio of 1:1. Bars show mean proliferation of CL4 cells at 48 h as counts per minute (cpm) of ^{3}H-thymidine incorporation. Error bars represent standard deviation (SD). Statistical analyses were carried out using one way ANOVA followed by Bonferroni's test, ***$P < 0.0001$. Data is representative of four separate experiments. **(C)** Groups of 6 to 8 week old BALB/c mice, were injected s.c. with either 1 × 10^6 RencaHA/T3 (•) or with RencaHA/T3/ICAM-1 (×) cells and tumor size plotted for each mouse (where n=5 for each group), and **(D)** mean size and SD for each group. Statistical analyses were carried out using one way ANOVA followed by Bonferroni's test, ***$P < 0.0001$